

Introduction

Certain populations such as chemotherapy patients and atomic bomb survivors have been exposed to ionizing radiation and experience tissue damage and cancer initiation and progression. One cancer that can be initiated from radiation is esophageal squamous cell carcinoma (ESCC), an epithelial cancer that has a survival rate as low as 20% (Chunping et al., 2009). Researchers have found that when protein tyrosine kinase receptors (RPTK) activate oncogenes, they can create epithelial tumors and cause deadly cancers like ESCC (Tannock et al., 2005). The RPTK family has one group, MET, that has only two receptors, MET and RON, present in the human body. MET's ligand is the hepatocyte growth factor (HGF) and RON's ligand is the macrophage-stimulating protein (MSP-1). Both HGF and MSP-1 have been shown to activate their receptors and are implicated in certain processes [What processes are these?] (Wang et al., 2003). Since radiation damages cells throughout the biological system (Camphausen et. al, 2001), researchers are investigating whether or not HGF and MSP-1 protects or kills certain normal and cancerous cells by being part of cell recovery processes.

One research group recently reviewed that the HGF-MET pathway has an important role in the embryonic development in the liver, migration of myogenic precursor cells, regulation of epithelial morphogenesis and growth, and regeneration and protection in tissues (Nakamura et. al, 2011). In addition, since the RON receptor is more commonly expressed in cells of epithelial origin, and when activated is part of epithelial cell matrix invasion, dissociation, and migration processes, scientists conclude that RON might be one of the factors causing epithelial cancer initiation in the biological system (Wang et. al, 2003). In order to examine HGF and MSP-1's effect on cancer initiation and progression we used two immortalized esophageal epithelial cell lines. One is a normal human cell line (EPC2-hTERT), while the other had a p53 mutation at the 175th amino acid position (EPC2-hTERT-p53^{R175H}). For this investigation, we used 0(control), 2, and 4 Gray doses of gamma (Cs¹³⁷) radiation and selected various concentrations from 0-100 ng/mL of HGF and MSP-1 in our assays.

Since the HGF and MSP-1 pathways have proliferative roles in epithelial cells, we conducted the MTT proliferation assay to see if either drug enhances or inhibits cell proliferation over time. Also, a MTT cytotoxicity assay was necessary to observe whether the drugs are protecting the cells from radiation and if a trend is occurring depending upon the amount of dose added. In addition, a wound healing assay was done since both drugs have been known to promote cell motility. Since cell damage occurs when radiation is added, apoptosis and micronuclei assays are vital to see if HGF and MSP-1 increase or decrease cell death and damage in normal and pre-cancerous cells and by how much based on the radiation dosage. Overall, we used the MTT, wound healing, apoptosis and micronuclei assays to investigate the effects of HGF and MSP-1 on irradiated esophageal epithelial cells.

Materials and Methods

- **Cell Lines:** Normal (EPC2-hTERT) and mutated (EPC2-hTERT-p53^{R175H}) esophageal epithelial cell lines were used this investigation. The cells were cultured and maintained according to the Phelan, 1998 cell culture protocol.
- **Radiation Dosages:** The gamma (Cs¹³⁷) radiation doses ranged from 0-4 Gys. (0-400 rads).
- **Drugs:** Hepatocyte growth factor (HGF) and macrophage-stimulating protein (MSP-1) concentrations ranged from 10-100 ng/mL depending on the assay.
- **Incubation Settings:** 37° Celsius, 5% CO₂, 98% humidity
- **Controls:** No drug or cells with KBM (keratinocyte basal medium)
- **4 assays:** MTT Proliferation and Cytotoxicity, Wound Healing, Micronuclei assay, and Apoptosis Assay

1. MTT Proliferation and Cytotoxicity

Materials:

- 7 (4 for proliferation and 3 for cytotoxicity) 96-well plates
- MTT Solvent: 4mM HCl, 0.1% Nandet P-40 (NP40) all in isopropanol
- MTT Reagent*: (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)
- PBS solution
- Aluminum/tin foil
- TECAN Spectrofluorometer

*Reagent is prepared with a concentration 5 mg/mL in PBS and is covered with foil

Methods:

1. Culture the cells (2000 cells/well) with KSFM (keratinocyte serum free medium) in the plates and incubate for 24 hours.
2. Add HGF and MSP-1 (10, 50, and 100 ng/ml) in each well according to the plate setup of both proliferation and cytotoxicity assays. Incubate again for 24 hours.

Proliferation:

1. Take one plate for the 24 hour reading and add 20 μ L of the 5 mg/mL MTT Reagent per well. After putting it on the shaker for a few minutes, incubate it for 3.5 hours.
2. Remove the media from the wells and add 150 μ L MTT solvent per well quickly since the plate is photosensitive. Put it on the shaker for 15 minutes.
3. Read the plate by using the TECAN spectrofluorometer.
4. At 48, 72, and 96 hours, repeat the same steps taken for the 24 hour reading.

Cytotoxicity:

1. Irradiate one plate each at 0, 2, and 4 Gray doses.
2. After 72 hours, repeat the same steps taken for the 24 hour proliferation plate for the 3 cytotoxicity plates.

Analysis:

To test how many live cells were present over the 96-hour or 72-hour period, we measured the intensity of the purple formazan color, which is produced when living mitochondria converts the yellow MTT reagent to purple by using enzymes during the 3.5 hour incubation period. The TECAN spectroflorometer then uses an absorbance at an optical density of 590 nm to measure this intensity and give values. For the proliferation assay, we can observe if HGF and/or MSP-1 promotes cell proliferation without any stress added to the experiment. In the cytotoxicity assay, we are adding the radiation factor to see if at one particular time if either drug protects both types of cells (Wallert and Provost Lab, 2007).

2. Wound Healing**Materials:**

- 3 24-well plates (2 with no radiation and 1 with a dose of 2 Gray)
- Camera
- PBS solution
- 200 μ L pipette tip
- Marker with a visible color as biomarker
- Media that contains KBM with HGF or MSP-1 (both drugs at 50 ng/mL)

Methods:

1. Draw a line with a marker on the bottom of each well

2. Use the tip to scratch once on each well to create a wound
3. Rinse the whole plate of cells with PBS solution
4. Replace the PBS solution with 1.5 mL of media containing the drug
5. Take a picture of each well at 0, 3, 6, 9, 12, and 24 hrs.

. [This is not the case – media was left on the whole time]

Analysis:

We used Image J (NIH) to measure the length of the wound from each picture taken**. After drawing two lines parallel to each other where the cells are bordered and one line connecting both perpendicularly, the software measures the length of the gap between the two sides of cells. We can then plot these values and find the percentage of the wound healed. For example, if 100% is the percentage at 24 hours, it means that the drug facilitated the cells to migrate and fill the gap completely over 24 hours. The wound assay can help us determine whether HGF and MSP-1 promote cell motility (Wallert and Provost Lab, 2004).

**Error: One picture of the p53 plate at 9 hours was missing.

2. Micronuclei Assay

Materials:

- (6) 4-chamber well slides
- 1X PBS [Wash buffer]
- Carnoy's fixative: 3:1 methanol : acetic acid
- DAPI in PBS (1:10 dilution working stock; 1:3000 dilution final)
- SlowFade Gold with DAPI

- Oil immersol with dropper
- OLYMPUS AX70 Upright Compound Microscope

Methods:

Preparing slides:

1. After culturing and seeding the cells, add HGF or MSP-1 (50 ng/mL) to their selected wells after 1 hour. Then wait for 24 hours.
2. Irradiate 2 slides (2 Gy.) and another 2 (4 Gy.), leaving 2 slides as control with no dosage.
3. After 48 hours, fix the cells in Carnoy's fixative.
4. Wash with PBS three times, with each for 5 minutes. Add DAPI solution (350 μ L/well) and incubate the slides for 5 minutes. Wash the cells again with PBS three times (five minutes each).
5. Mount each slide by adding 3-4 drops of SlowFade Gold and using a 22x50 mm cover glass. Seal each slide with nailpolish on the outer boundary.

Counting:

1. Turn on the microscope.
2. Add one drop of oil immersol on Coverslip.
3. Touch the 100x objective lenses with the oil and turn on the fluorescence blue light
4. Count the number of micronuclei and binucleated cells.



Picture 1: A field of view showing 1 binucleated cell with one micronuclei.

Analysis:

When the fluorescence light is turned on, we counted the blue cells that are binucleated and also the ones that have micronuclei (Picture 1). We analyzed the percentage of micronuclei of all the binucleated cells to find quantitatively how much damage occurred since micronuclei is composed of DNA fragments that are all clumped together. (Genpharmtox).

3. Apoptosis Assay

Materials:

- (6) 4-well chamber well slides
- 1X PBS [Wash buffer]
- 4% paraformaldehydeProlong Gold with DAPI
- 0.5% Triton (Permeabilizing buffer)
- 3% BSA (Blocking buffer)
- Primary antibody solution (Cleaved caspase-3, 1:300 dilution in blocking buffer)
- Secondary antibody solution (Alexa-Fluor 488 goat anti-rabbit IgG antibody, 1:1000 in blocking buffer)

Methods:

Preparing slides:

1. After culturing and seeding the cells, add HGF or MSP-1 (50 ng/mL) to each selected well (based on plate setup) after 1 hour.
2. After 24 hours, irradiate 2 slides with 2 Gray while and another 2 with 4 Gray, leaving two as control with no dosage. After 48 hrs, wash with PBS and fix the cells with 4% paraformaldehyde for 10 min. at room temperature. ***
3. Wash the cells three times for five minutes each with PBS.

4. Add 400 μ L/well permeabilizing buffer for 3 minutes at room temperature. Add 400 μ L blocking buffer to each well and incubate for 30 minutes to 1 hour.
5. Add primary antibody solution and incubate for 1-2 hours at room temperature. Wash three times for five minutes each with PBS.
6. Next, add secondary antibody solution (150 μ L/well) and incubate 30 minutes to 1 hour at room temperature without being exposed to light.
7. Wash three times for five minutes each with PBS. Use 3-4 drops of Prolong Gold with DAPI/slide, and use 22x50mm cover glass to mount and seal each slide with nail polish.

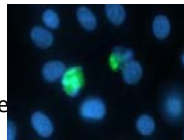
Counting: same as in Micronuclei assay except:

1. Touch the 60x objective lenses with the oil and switch between the blue & green lights
2. Count positive (green) cells/1000 blue (normal) cells = apoptotic index.

***If storing, replace paraformaldehyde with PBS and cover with parafilm, and store it at 4° C.

Analysis:

For this assay, we counted the positive



Picture 2: A field of view showing 2 positive apoptotic cells and many other blue cells.

if any cleaved caspase-3 enzymatic activity occurred. For every field of view, we counted the number of blue cells first while the blue light is on. Then, we switched to the green light to see if any positive cells were there in the same field of view. If there is at least one bright green cell, we switched back to the blue light to see if a blue cell is overlapping in the same position to clarify if it actually a cell (Picture 2) (Promega, 2011).

Result

Picture 2: A field of view showing normal (blue) and two apoptotic (green) cells.

1. MTT Proliferation and Cytotoxicity

Proliferation: The MTT proliferation assay was conducted with 10, 50, or 100 ng/mL concentrations of HGF and MSP-1 in both normal and mutated cell lines over a 96-hour period. The results present that cell proliferation increased over time in both normal and mutated cells. Interestingly, there was no positive or negative effect in both cell lines as the concentrations of either drugs (10, 50, and 100ng/mL) increased. When considering HGF and MSP-1, our results show that both drugs do not aid in cell proliferation in both normal and mutated epithelial cells (Figure 1.1a & 1.1b).

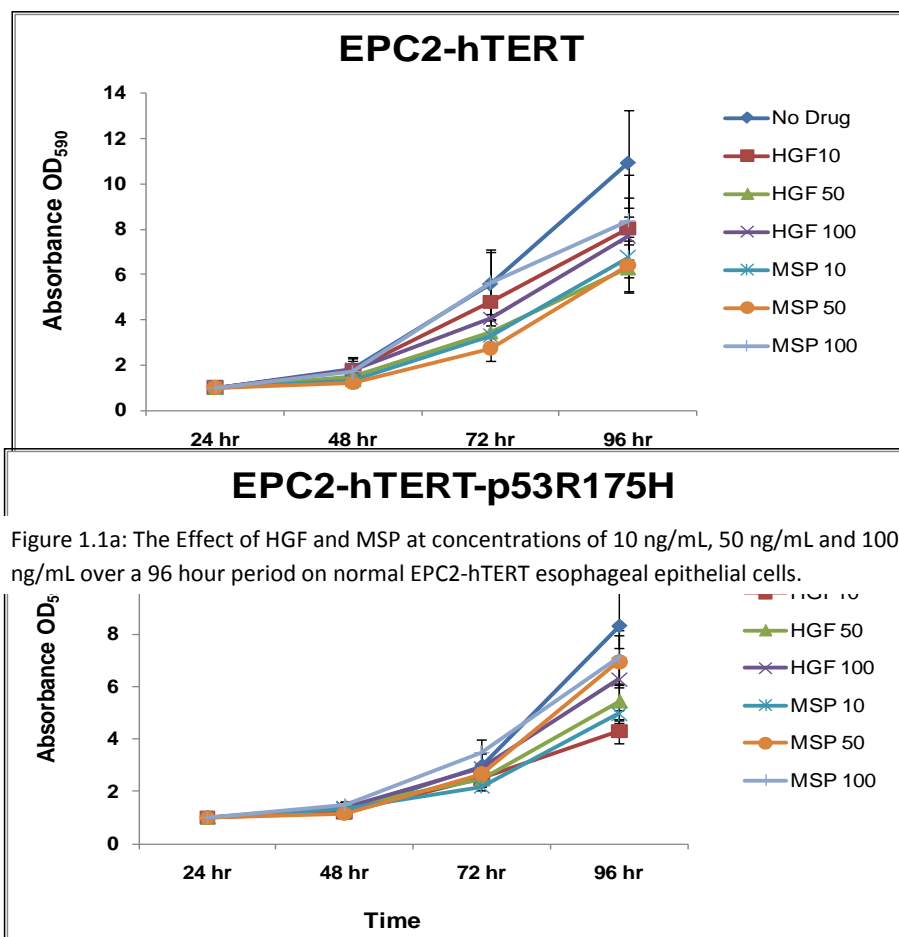


Figure 1.1b: The Effect of HGF and MSP at concentrations of 50 ng/mL and 100 ng/mL over a 96 hour period on mutated EPC2-hTERT-p53^{R175H} esophageal epithelial cells.

Cytotoxicity: For the MTT Cytotoxicity assay, the cells were given 2 and 4 Gray dosages of gamma radiation with two HGF and MSP-1 (50 or 100 ng/mL) concentrations. The results presented in Figures 1.2a & 1.2b show that as radiation dosage increased in both cell lines with no drug, the number of EPC2-hTERT decreased significantly while there was little change in the p53-mutated cells. When considering HGF and MSP-1's effects, they interestingly protected the normal cells more than the mutated cells from radiation (Figure 1.2a & 1.2b).

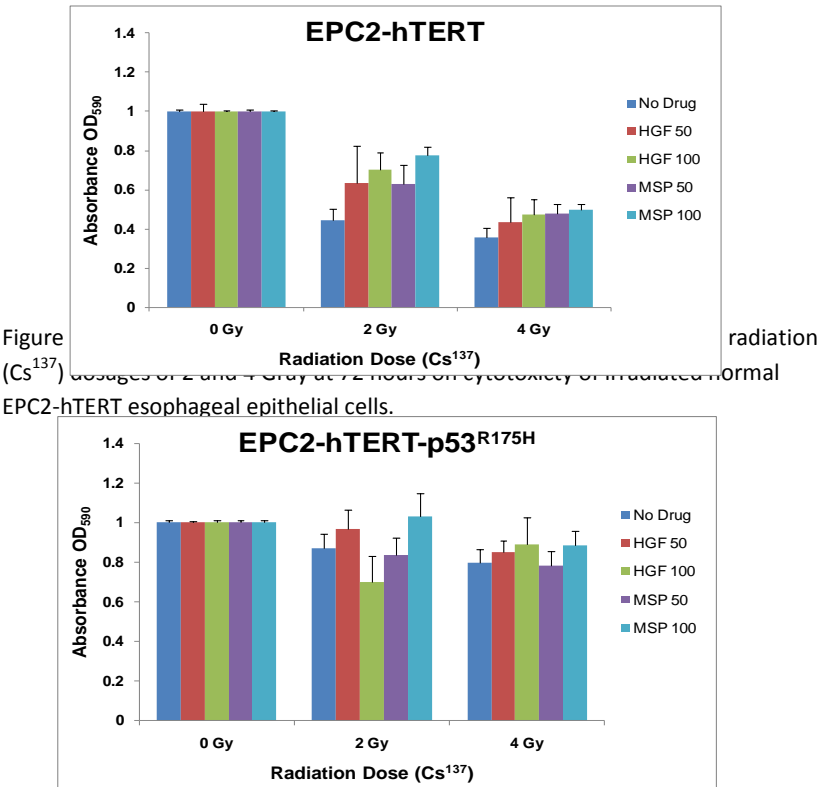


Figure 1.2b: The Effect of HGF and MSP (50 and 100 ng/mL) with gamma radiation (Cs¹³⁷) dosages of 2 and 4 Gray at 72 hours on cytotoxicity of p53-mutated esophageal epithelial cells.

2. Wound Healing: This assay allowed us to observe how much normal and mutated cells migrated with 50 or 100 ng/mL concentrations of HGF and MSP-1 over a 24 hour period. According to the results shown in Figures 2.1 & 2.2, both HGF and MSP-1 enhanced cell migration more than the cells with only KBM after 24 hours. To compare overall how much the normal and mutated cells migrated with the KBM control, HGF, and MSP-1 factors, Figure 2.3 is shown after 12 hours of healing. From Figure 2.3, HGF seems to be an effective drug in enhancing cell migration in normal cells than in the mutated cells since HG

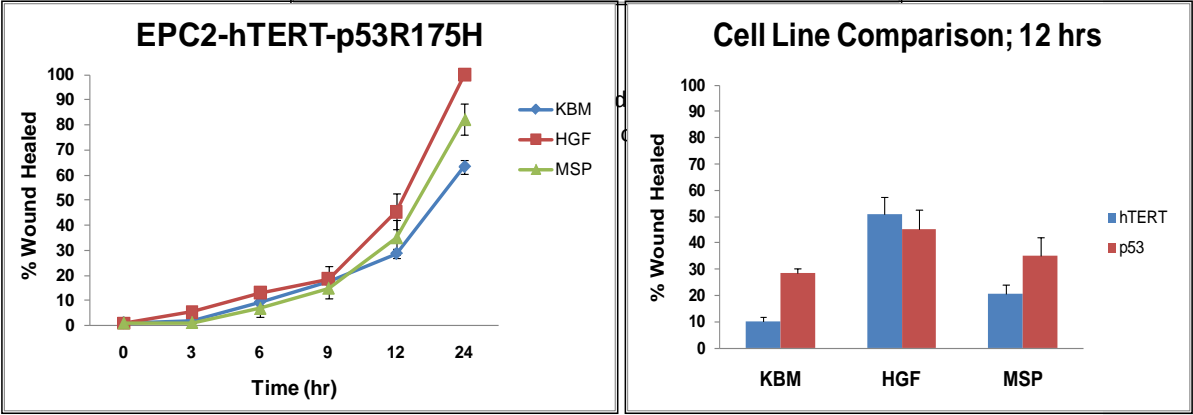
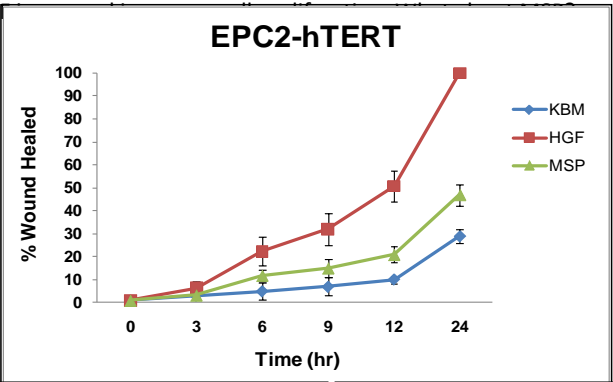
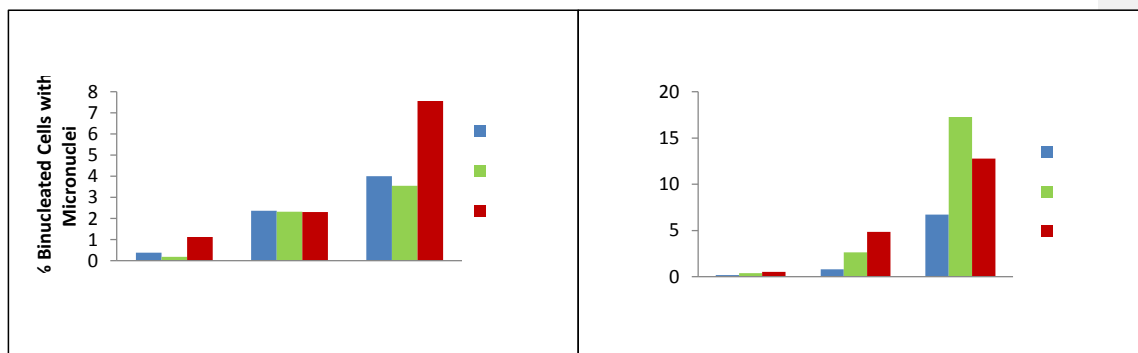


Figure 2.2: The Effect of HGF and MSP at concentration of 50 ng/mL over a 24 hour period on mutated EPC2-hTERT-p53^{R175H} esophageal epithelial cells.

Figure 2.3: The Effect of KBM control, HGF and MSP (50 ng/mL) at 12 hours on mutated EPC2-hTERT-p53^{R175H} esophageal epithelial cells.

3. **Micronuclei Assay:** The cells were irradiated at 2 and 4 Gray with 50 or 100 ng/mL concentrations of HGF and MSP-1. In both figures, radiation seems to cause significant cell damage since there was an increase in the percentage of binucleated cells with micronuclei in both cell lines as the radiation dosage increased. Specifically for HGF, more micronuclei formed in both types of cells except at 4 Gray for the mutated cells. Also, at 2 Gray, both HGF and MSP-1 did not enhance micronuclei formation activity in normal cells as much as in the mutated cells (Figures 3.1 & 3.2).



4. **Apoptosis Assay:** In this assay, we added 50 or 100 ng/mL concentrations of HGF and MSP-1 to both cell lines and counted them after 48 hours of radiation exposure (2 or 4 Gray). According to the results shown in Figures 4.1 & 4.2, as radiation dosage increased, the apoptotic activity (programmed cell death) increased in both cell lines in

respect to the drug added. Without any radiation dosage, HGF and MSP-1 enhanced apoptosis (Figures 4.1 & 4.2). Specifically in Figure 4.1, HGF had no/little effect while MSP-1 significantly inhibited cell death in the normal cells. In the mutated cells, even though there was a little increase in apoptosis at 2 Gray, both HGF and MSP-1 enhanced cell death at 4 Gray. MSP-1 in particular, caused more apoptosis than HGF (Figure 4.2).

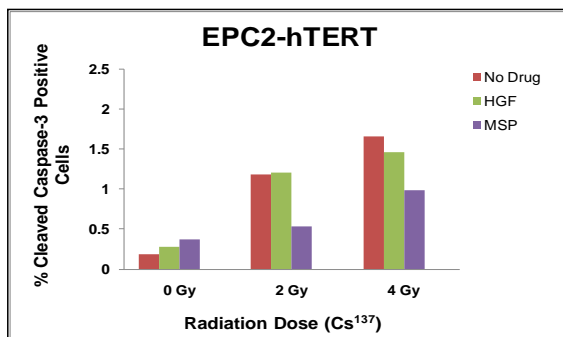


Figure 4.1: The Effect of HGF and MSP at concentration of 50 ng/mL on normal EPC2-hTERT esophageal epithelial cells with 0, 2, or 4 Grays of Cs¹³⁷ gamma

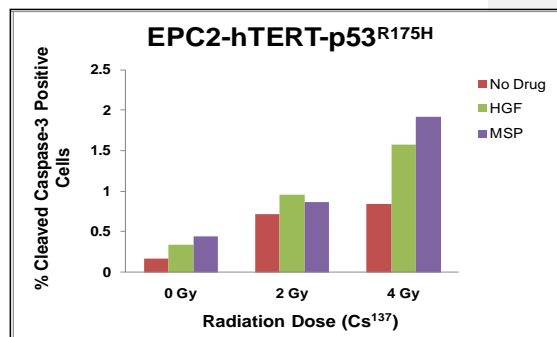


Figure 4.2: The Effect of HGF and MSP at concentration of 50 ng/mL on mutated EPC2-hTERT-p53^{R175H} esophageal epithelial cells with 0, 2, or 4

Discussion

Our MTT proliferation assay results showed that neither HGF or MSP-1 cause cell proliferation in normal and mutated epithelial cells. At these concentrations and times tested, the growth factors may not promote proliferation. Additional explanations include several errors such as possible bacterial contamination, not protecting the plates from light, time misalignments, and pipetting errors might have caused this. In the future, conducting more runs might give better and consistent results with previous work that has already been done on this assay for HGF and MSP-1.

In the MTT test for cytotoxicity, the normal cells had significant changes in samples with and without drug as radiation dosage increased. This supports earlier reports that HGF and MSP-1 have protective properties on cells from stress. (Nakamura et. al, 2011) (Wang et. al, 2003). When HGF or MSP-1 was added, they had significant protection, especially the cells with MSP-1 (100 ng/mL). For the p53-mutated cells, there was no effect of radiation and there were little differences even among the cells with HGF or MSP-1. Cancer cells are known to replicate fast, especially with the well known p53 mutation. This mutation prevents a cell with DNA damage from undergoing apoptosis, thus allowing it to proliferate. This explains why the mutated cells with no drug did not die easily (Vogelstein et al., 2000). If the mutated cells are already protecting themselves, then the drugs' intervention would not make much difference, which is clearly shown in the figures. Additionally, if the cells are already programmed to proliferate, then the 2 and 4 Gray doses may not be enough to reduce the number of cells and observe if HGF and MSP-1 take part in protecting the cells. If the mutated cells are much more aggressive than the normal cells, then in the future, higher doses may be used to see better results.

The wound healing assay showed that both drugs enhanced migration in both cell lines. There were positive results in both figures, but might give contradictory information when considering the drugs for chemotherapy purposes. HGF and MSP-1's migration activity was higher than the KBM control's after 24 hours for both cell lines. Previous research has shown that HGF's effect in epidermal keratinocytes (Nakamura et. al, 2011) and MSP-1's intervention in epithelial cancers (Wang et. al, 2003) resulted in similar results. In the normal cells, HGF addition resulted in 100% wound healing and MSP-1 addition resulted in 47% wound healing.

Comment [ZP1]: Do not use words like "successful" or "unsuccessful" in terms of experiments and data. The data is what it is, neither "right" nor "wrong"

In the mutated cells, HGF's still 100% and MSP-1's (83%) higher percentage of wound healed show that recovery of cancerous cells is occurring. MSP-1 appears to have a greater motogenic effect on p53-mutated cells compared to normal epithelial cells, indicating that pre-cancerous cell lines are more sensitive to external cues that can lead to migration and invasion (hallmarks of cancer) (Ludwig Institute for Cancer Research). Future studies on how HGF and MSP-1's affects other epithelial cancers may show if the trends found in this project is only specific to esophageal squamous cell carcinoma.

In the micronuclei assay, both figures show that as the radiation dosage increased, cell damage increased for the control, HGF, and MSP-1 in both cell lines. Without considering the difference in the scales in both figures, all cells with either/no drug increased the percentage of micronuclei at 4 Gray compared to the other dosages. Also, considering the difference in the scales, there was more cell damage occurring in the mutated cells than in the normal cells. This might explain the fact that since all the cells are still living, the radiation causes significant damage on cells that do not have cell repair processes that work properly (NIH, 1999). For the normal cells, HGF enhanced more cell damage at the highest dose, showing that HGF may not be advisable for future chemotherapy treatments. For MSP-1, one study supported our results that MSP-1 had a neutral effect on the normal cells (Sifa, 2009). On the other hand, MSP-1 caused more cell damage than HGF at the highest dose in the mutated cells, suggesting that MSP-1 could be investigated further as a potential drug in cancer research. Even though HGF and MSP-1 caused certain trends in both cell lines, more runs on this assay should be done to confirm our observations that may account for counting errors and possible contamination with the cells. Lastly, both figures present that more micronuclei are forming in the mutated cells

than the normal cells (see the scales closely) no matter which drug is used. This observation is important since this assay involves with only live cells, it suggests that the pre-cancerous cells are surviving with cell damage that can cause further deterioration on the human esophagus. Under normal circumstances, the cells that are having significant amounts of damage should be undergoing apoptosis (NIH, 1999); however, the p53 mutation prevents this and clearly shows that further investigation on this matter is vital (Vogelstein et al., 2000).

In general, as radiation dosage increased, more apoptotic activity occurred (Camphausen et. al, 2001). Even though HGF did not affect the normal cells in any way, the drug promoted apoptosis in the mutated cells (especially at 4 Gray). On the other hand, MSP-1 inhibited apoptosis in the normal cells significantly while promoting apoptosis at 4 Gray in the mutated cells. This supported by a research group's findings that MSP-1 is known to help epithelial cells survive since there was less than 10% apoptotic activity in normal epithelial cell cultures than in cultures with only media (Danilkovitch et. al, 1999). HGF and MSP-1 both seem to show opposites trends again, as seen in the micronuclei assay. To confirm HGF's neutrality on irradiated normal cells, higher doses should be used in the future to see if that is not the case. Also, even though MSP-1 may be thought as a possible drug in future cancer treatments, MSP-1 did not show major differences at 2 Gray for the mutated cells. More runs on this assay may be necessary to see if MSP-1 is only effective to promote apoptosis in ESCC at 4 Gray dose.

Conclusion

There were many factors used to investigate HGF and MSP-1's activities. We used radiation as an environmental stress and found that the drugs may be most active only at certain dosages. We have found that while 2 and 4 Gray doses are sufficient to cause protection in the normal

cells, HGF and MSP-1 seem to need more dosages of radiation have an effect on the p53 cells. When forming micronuclei, both drugs enhanced more cell damage in the p53 cells than the normal cells, with MSP-1 only causing more at 4 Gray in the p53 cells than the normal hTERT cells. In terms of apoptosis, both drugs caused much damage at 4 Gray in the cancerous cells. Also, MSP-1 prevented significant cell death as the radiation dosage increased in the normal cells. Since radiation has brought both short and long term effects on astronauts in the past decades, current investigation is occurring now in finding out the role of certain growth factors in radiation-induced esophageal carcinogenesis..